

CHARACTERIZATION OF CISTRON SPECIFIC FACTORS FOR THE INITIATION OF MESSENGER RNA TRANSLATION IN *E. COLI*

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1. Introduction

Comparison of the translation of different mRNAs in cell-free systems from *E. coli* has shown the function of initiation factor* IF3 in mRNA selection by ribosomes [1–3]. This initiation factor activity can be separated into several fractions differing in their relative activity for the translation of T4 mRNA as compared to MS2 RNA. The change in template specificity of ribosomes after T4 phage infection [4, 5] was also accounted for by a variation in IF3 activity [6]. Further an IF3 fraction, which was purified to homogeneity, selectively stimulated on MS2 RNA the attachment of ribosomes to the coat protein cistron initiation site [3]. This implied that the initiation sites of the different cistrons of such a phage RNA [7] are recognized by different factors and we report here the separation of MS2 RNA cistron specific IF3 fractions. The molecular basis for this heterogeneity of IF3 activity is, however, not yet clear.

Recently, we have identified new protein factors associated with IF3 activity which influence the mRNA recognition process. Such proteins have no initiation activity, unless complexed with the 22,000 molecular weight IF3 protein and might at least, in part, be responsible for the functional heterogeneity observed. Variations in the cell activity to translate different mRNAs could result from changes in either IF3 itself or in the associated factors.

* Initiation factors activities of *E. coli* are from now on designated IF1, IF3 and IF2 instead of F1, F3 and F2 [12] or A, B and C [8], respectively.

2. Materials and methods

Crude initiation factors were prepared from the 1M NH_4Cl ribosomal wash fluid [2] from 1200 g *E. coli* MRE 600. The proteins precipitated by 80% saturation of ammonium sulfate were dissolved in buffer P: 20 mM potassium phosphate buffer pH 7.2, 0.2 mM MgCl_2 , 7 mM β -mercaptoethanol, 5% glycerol and initiation factor activities IF1 (A), IF3 (B) and IF2 (C) separated by DEAE-cellulose chromatography (fig. 1). Published procedures were used to obtain purified IF1 and IF2, formyl ^{35}S -methionyl-tRNA as well as ribosomes and supernatant free of initiation factors as before [8, 9]. T4 mRNA from a 20 min T4 infected culture of *E. coli* [10] and RNA from phage MS2 [11] were used to measure IF3 activity as before [2]. Fractionation of IF3 activities is described in the text.

3. Results and discussion

3.1. Separation of mRNA and cistron specific fractions of IF3 activity

Crude initiation factor fraction was chromatographed on a DEAE-cellulose column developed with a gradient of potassium phosphate buffer pH 7.2 from 0.02–0.3 M. A typical pattern of elution is shown in fig. 1. Initiation factor IF2 activity is followed by measuring fmet-tRNA binding to ribosomes [8] and IF3 by assay of T4 mRNA translation in the presence of purified IF1 and IF2 [2]. IF1, which is not retained on DEAE-cellulose, is not shown in fig. 1.

The region corresponding to IF3 (fraction 320–420 in fig. 1) was pooled and rechromatographed on a

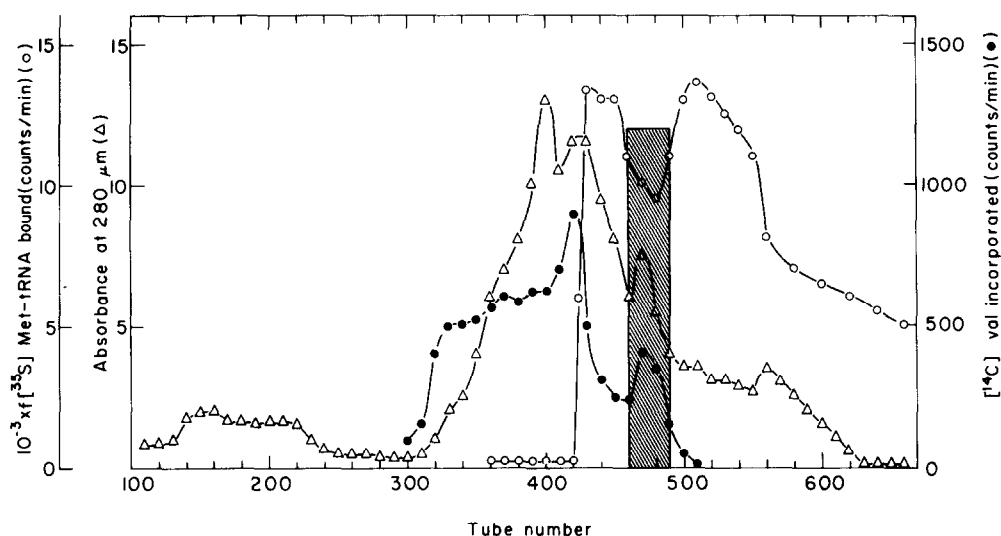


Fig. 1. DEAE-cellulose chromatography of crude initiation factors. In this particular experiment crude factors (50 g protein) from 5000 g *E. coli* MRE 600 were chromatographed on a 6.6 × 78 cm DEAE-cellulose (Serva) column equilibrated in buffer P. Fractions of 20 ml were collected and assayed as described in the text. Elution pattern of IF3 activity (●—●—●) and IF2 activity (○—○—○) are shown. The hatched region was the source of factor i (see fig. 3).

column of DEAE-Sephadex A-50. At this stage IF3 activity is assayed using both T4 mRNA and MS2 RNA translation and fractions exhibiting different translation ratios for the 2 mRNAs are separated as shown by table 1. The separation is always somewhat arbitrary since no clearly spaced peaks are obtained. Nevertheless, these IF3 fractions can be further purified

as indicated in table 1, and yield purified IF3 activities with high specific activities and very different T4/MS2 RNA translation ratio. Fraction IF3-B2 was found to be homogeneous at this stage. It appears (fig. 3) as a small basic polypeptide chain with a molecular weight of about 22,000, very similar to the protein purified by Sabol et al. [12].

Table 1
Fractionation of IF3 activity on DEAE-Sephadex.

NH ₄ Cl concentration of eluate (M)	Nomenclature	¹⁴ C-Valine incorporation with			Specific activity ¹⁴ C-valine incorporated with T4 mRNA (pmoles per μg protein)
		MS2 RNA (cpm)	T4 mRNA (cpm)	T4 mRNA MS2 RNA	
0.05	F3.B1	879	1695	1.9	32
0.10	F3.B2	1120	1500	1.3	86
0.15	F3.B3	439	1657	3.8	70*
0.20	F3.B4	25	780	31.0	21**
—	Crude factor	—	—	—	0.35

DEAE-cellulose fractions corresponding to IF3 activity (1,500 mg protein) were rechromatographed on a 1.5 × 34 cm column of DEAE-Sephadex A50 with a 0.02–0.3 M NH₄Cl gradient in the buffer P. Each fraction was assayed for its activity to stimulate translation of MS2 RNA and T4 mRNA [2]. 4 fractions were obtained. F3.B3 was further purified on hydroxylapatite from which it was eluted with 0.3 M phosphate buffer (Buffer P). F3.B4 was further purified on phosphocellulose from which it elutes at 0.4 M phosphate buffer P. In the assays a background incorporation with IF1 + IF2 alone of 100 cpm was subtracted with both mRNAs.

* Further purified on hydroxylapatite. ** Further purified on phosphocellulose.

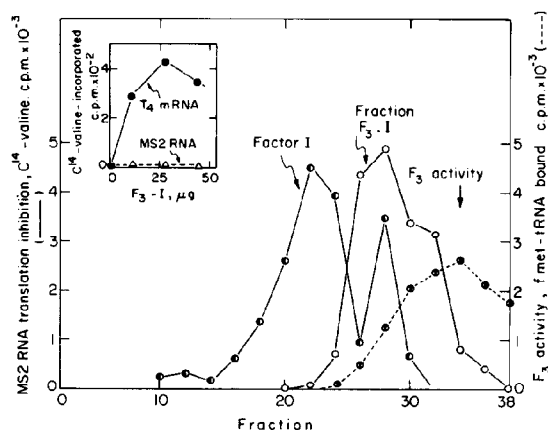


Fig. 2. Isolation of a natural IF3-i complex. Sedimentation pattern on a 5-15% glycerol gradient in 0.02 M potassium phosphate buffer pH 7.2, 1 mM Mg acetate, 0.01 M NH_4Cl , 1 mM DTT, of purified factor i (see fig. 3) alone ($\bullet-\bullet-\bullet$), or purified IF3 alone (position of peak activity shown by arrow) or of an IF3-i complex obtained during the fractionation of IF3 activity in the following way: from the DEAE-cellulose chromatography shown in fig. 1, tubes 320-360 were pooled and rechromatographed on a DEAE-Sephadex A-50 column, IF3 activity eluted from this column at 0.02 M NH_4Cl in buffer P was further purified on a Phosphocellulose Whatman P11 column from which it was eluted at 0.6 M NH_4Cl in buffer P. At this stage this IF3 activity assayed as in table 1 appeared specific for T4 mRNA translation as shown in the insert. An aliquot of 220 μg of this IF3 fraction was analyzed by gradient centrifugation in a Spinco SW 50.1 for 16 hr at 40,000 rpm. Each fraction was assayed for IF3 activity by measuring the stimulation of fmet-tRNA binding to 70 S ribosomes [9] ($\circ-\circ-\circ$), and for factor i activity by measuring the inhibition of native MS2 translation as in table 3. ($\circ-\circ-\circ$). ^{14}C -valine incorporation in the control without factor i was 6000 cpm.

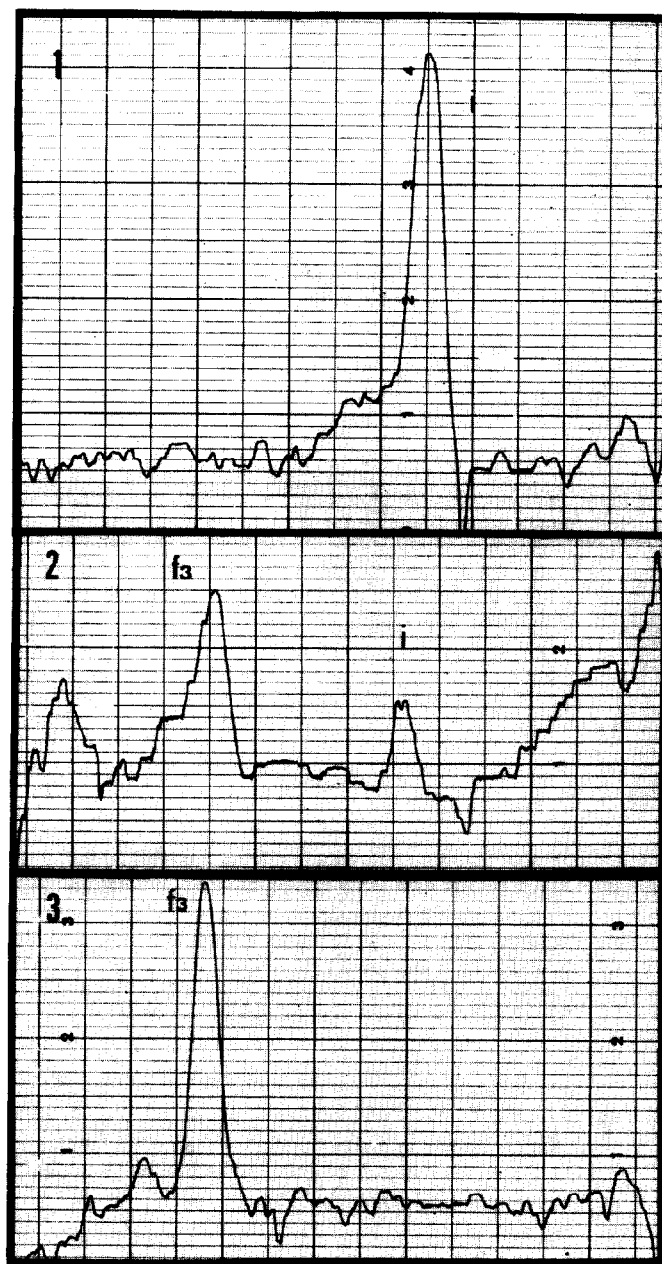
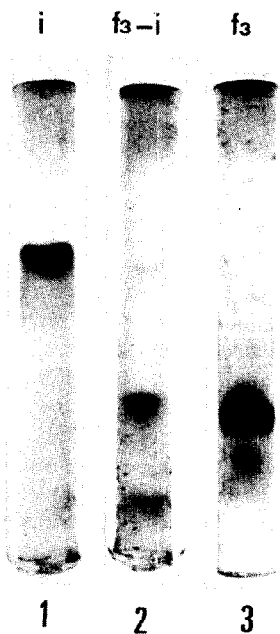


Fig. 3. Acrylamide gel electrophoresis in SDS of initiation factor IF3, factor i and IF3-i complex. Factor i activity is assayed by measuring the inhibition of native MS2 RNA translation as in table 3. Factor i was purified from DEAE-cellulose chromatography fraction shown in fig. 1 by DEAE-Sephadex hydroxylapatite and finally by glycerol gradient centrifugation (Gröner et al., in preparation). 7 μg factor i were submitted to SDS gel electrophoresis according to Weber and Osborn [16] in gel number 1. Gel number 2 shows the analysis of the fraction IF3-i obtained from glycerol gradient shown in fig. 2 (tubes 26-30) and which represents a natural complex between initiation factor IF3 and factor i. In gel number 3, 14 μg of IF3.B1 fraction (table 1) were analyzed. Scanning of the gels stained with Coomassie blue is shown in the figure.

Table 2
Translation of MS2 RNA.

Net stimulation with factor:	³⁵ S-formylmethionine labeled peptides			Ratios Coat Synthet
	I (Coat) (cpm)	II (Synthet) (cpm)	III (cpm)	
F3.B1	2700	4400	550	0.6
F3.B2	1500	500	250	3.0
F3.B3	1100	1100	350	1.0

(HCHO)—MS2 RNA, 50 µg, was incubated in 0.2 ml protein synthesis reaction mixture [8] with 370 µg ribosomes, 5 µg IF1, 12 µg IF2, 17 µg F3.B1, 8.5 µg F3.B2, or 7.5 µg F3.B3 (see table 1), 15 µl of supernatant and 7.5×10^5 cpm ³⁵S-fmet-tRNA (4000 cpm/pmole). After 20 min at 37°, proteins were acid precipitated and submitted to trypsin and chymotrypsin fingerprinting according to Lodish [13]. After electrophoresis at pH 1.9, on thin layer DEAE-cellulose plates, radioactivity in each peak was computed. Incorporation with IF1 and IF2 alone (I = 1100; II = 3500 and III = 200) was subtracted. Peptides were identified by further pronase digestion: peptide I yielded only fmet and fmet ala while peptide II yielded fmet, fmet ser and small amounts of deformylated products.

Table 3
Factor i activity.

Conditions	Formyl- ³⁵ S-methionine incorporation		
	T4 mRNA (cpm)	MS2 RNA (cpm)	MS2 RNA (formaldehyde treated) (cpm)
Control	36,700	11,300	13,800
+ factor i, 5 µg	38,500	4,200	15,200

80 µg T4 mRNA, 16 µg MS2 RNA or formaldehyde treated [14] MS2 RNA were incubated 30 min at 37° in 0.05 ml reaction mixtures [8] with 3 µg IF1, 15 µg IF2 and 0.5 µg IF3, 100 µg ribosomes, 5 µl high speed supernatant and 5 µg of formyl-³⁵S-methionyl-tRNA (12,500 cpm per µg) to measure amino terminal labeling of the protein product. A background of 2000 cpm incorporated in the absence of template was subtracted.

The activity of these fractions for the translation of the 3 cistrons of MS2 RNA was studied by fingerprinting the terminally labeled protein products, using formyl ³⁵S-methionyl-tRNA as source of radioactivity according to Lodish [13]. Only the amino terminal methionine of each protein is labeled and the trypsin—chymotrypsin radioactive peptides are separated by electrophoresis on thin layer cellulose plates at pH 1.9. The coat protein products remain close to the origin (peptides 1 in table 2) while the product from synthetase migrates faster (peptides 2). (A small amount of material migrates even further and might originate from the maturation protein.) Further degradation of the products with pronase and electrophoresis at pH 3.5 reveals that peptides 1 contain formylmethionylalanine (the beginning of the coat protein) and peptides 2, formylmethionylserine (the beginning of the synthetase)

in agreement with Lodish [13]. The purpose of our experiments was to search for differential effects of the IF3 fractions on the relative translation of the different cistrons of MS2 RNA. Since the secondary structure of the phage RNA has been shown by Lodish to restrict initiation to the coat protein cistron, the RNA was first treated with formaldehyde to allow independent initiation at both coat protein and synthetase cistrons [14]. Under these conditions, in a crude *E. coli* extract, more synthetase is made than coat protein. From the data presented in table 2 it is clear that the translation of the coat and synthetase cistrons are stimulated very differently by the 3 fractions of IF3 activity tested. Particularly F3.B2 appears to stimulate selectively coat protein synthesis. As reported elsewhere by Berissi et al. [3] direct studies on the RNA site to which ribosomes are bound showed that

F3.B2 stimulates selectively the attachment of ribosomes to the coat protein cistron initiation site. This clearly indicates that a homogeneous preparation of IF3 possesses mRNA selection properties. As shown in table 3, the other IF3 fractions, in contrast, stimulated translation of the synthetase cistron to a larger extent than the coat protein cistron. These results therefore confirm our previous reports [2,3] on the separation of IF3 activities responsible for the initiation of translation of different cistrons of MS2 RNA. Fraction F3-B1 contains an activity for initiation at the synthetase cistron which is absent in fraction F3-B2. These factors are therefore characterized by a different specificity toward T4 and MS2 RNA as well as toward the different cistrons of this phage.

3.2. Variations in mRNA specificity of initiation factors from *E. coli* in different physiological conditions

Ribosome associated factors obtained from *E. coli* MRE 600 can vary widely in their ability to support the translation of MS2 RNA relative to T4 mRNA. Crude factors prepared from actively growing *E. coli*, harvested rapidly in the logarithmic phase, usually exhibit a good MS2 RNA translation. We have, however, observed that crude factor prepared from massive cultures of *E. coli* (in a fermentor of 500 l for example) may be very poor in their activity for MS2 RNA. These preparations usually inhibit protein synthesis when used in large amounts. Further, crude factors from stationary phase *E. coli*, which show a lowered initiation factor activity [15] are much less active for MS2 RNA than for T4 mRNA.

These variations in uninfected *E. coli* cells are reminiscent of the situation observed after infection of *E. coli* by a phage such as T4 [4-6] where one obtains crude factors which appear devoid of IF3 activity for MS2 RNA while containing IF3 active for T4 mRNA [6].

In all these situations where the crude factors are almost inactive with MS2 RNA we have observed that upon fractionation of the crude factors on a DEAE-cellulose column similar to that presented in fig. 1, the IF3 activity for MS2 RNA translation could be shown to be present and could be recovered during the purification. This observation made with crude factor from both uninfected and T4 infected *E. coli* MRE 600, prompted us to look for additional factors in-

fluencing the initiation factor activity. Addition of the different fractions from the chromatogram to an MS2 RNA directed cell free system containing purified IF1, IF2 and IF3 showed the existence of several interfering activities, which changed the T4/MS2 RNA translation ratio.

3.3. Cistron specific interfering factors: factor i

We have undertaken to characterize these interfering factors and have identified at least one such activity eluting in the hatched region shown in fig. 1. This activity, which has now been purified to homogeneity and designated factor i (see fig. 3), appears responsible at least partly for the large inhibition of MS2 RNA translation observed in the crude factor as shown in table 3. Addition of this protein of about MW 70,000 to the cell-free system produces an inhibition of native MS2 RNA translation while apparently without effect on the overall amino acid incorporation directed by T4 mRNA. Strikingly, factor i also does not inhibit when formaldehyde treated [14] MS2 RNA is used as template. A more detailed study of the products of MS2 RNA translation, which will be presented elsewhere (Groner et al., in preparation), shows that factor i acts by inhibiting translation of the coat protein cistron while it stimulates translation of the synthetase cistron and this explains the apparent differential effect on native and formaldehyde treated MS2 RNA. Similarly, translation of some cistrons of T4 mRNA is inhibited, while that of others stimulated, by the addition of factor i.

Preliminary data suggest the existence of several i-like factors. It is important to stress that these interfering factors have no initiation activity by themselves and their effects are seen only in the presence of the purified initiation factors IF1, IF2 and IF3.

3.4. Heterogeneity in IF3 activity and the existence of IF3-i complexes

Indeed, factor i readily interacts with purified IF3 to form an IF3-i complex (Groner et al., in preparation). Moreover, one of the fractions of IF3, eluted from the DEAE-Sephadex (table 1) at concentrations of NH_4Cl below 0.05 M, appears to be a naturally existing IF3-i complex, containing both initiation factor F3 and factor i activities. This fraction was further purified on phosphocellulose column at 0.1 M NH_4Cl in buffer P on which it is retained while free factor i is

not. Analysis of this fraction is shown on fig. 2. It contains IF3 activity only for T4 mRNA as shown by the insert. As factor i (table 3) this fraction inhibits the translation of native MS2 RNA, but not of formaldehyde treated MS2 RNA. Upon analysis by glycerol gradient a region of overlapping IF3 and i activities was seen which sediments in the region between free i and free IF3 (IF3 normal position is shown by the arrow). This region when analyzed by SDS gel electrophoresis as in fig. 3 yields the typical IF3 and factor i bands. Complexes made by mixing purified factors IF3 and i sediment at the same position.

The different activities of IF3 fractions for the coat protein and synthetase cistron of MS2 RNA and for T4 mRNA (tables 1 and 2) could be explained by the existence of such an IF3-i complex. Purified IF3 (fig. 3) appears as a small basic protein of MW around 22,000 [12]. This polypeptide unit is present in all fractions exhibiting IF activity, even when they differ in their mRNA specificity (as in table 1). It is possible that detailed analysis of the primary structure of these molecules will show some structural differences. Until now, however, we have observed that an antibody prepared against IF3-B2 (table 1) cross reacts with all the other IF3 fractions (Scheps and Inouye, unpublished). Alternatively, it might be proposed that IF3 activity may be modified through interaction of the small basic polypeptide unit with cistron specific interfering factors such as factor i or similar proteins. In such a possibility the study of the role of these factors and their variations in the cell might become very important for understanding the regulation of mRNA translation.

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